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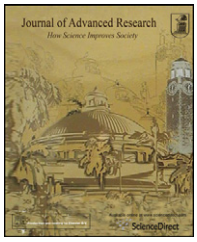

ORIGINAL ARTICLE

Isolation and molecular characterisation of malathion-degrading bacterial strains from waste water in Egypt

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KEYWORDS

Degradation;
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Bacillus thuringiensis;
Malathion;
Characterisation

Abstract Efficiencies of local bacterial isolates in malathion degradation were investigated. Five bacterial isolates obtained from agricultural waste water were selected due to their ability to grow in minimal salt media, supplied with 250 ppm malathion as sole source of carbon and phosphorus. The purified bacterial isolates (MOS-1, MOS-2, MOS-3, MOS-4 and MOS-5) were characterised and identified using a combination of cellular profile (SDS-PAGE), genetic make up profile (RAPD-PCR), and morphological and biochemical characteristics. Four bacterial isolates (MOS-1, MOS-2, MOS-3 and MOS-4) with identical genetic characteristics were identified as *Enterobacter aerogenes*, whereas isolate MOS-5 was identified as *Bacillus thuringiensis*. The degradation rate of malathion in liquid culture was estimated during 15 days of incubation for the isolate MOS-5 of *B. thuringiensis*. Slightly more than 50% of the initial malathion was decomposed within 3 days. The malathion concentration decreased to almost 17% in the inoculated medium after 10 days incubation, while more than 91% of the initial malathion was degraded after 15 days.

Introduction

Malathion is an organophosphate insecticide and acaricide that has been in use for some time as a DDT substitute for the control of field crop pests, household insects, flies and animal parasites [1]. Despite its high toxicity, malathion is still extensively used throughout the world [2]. In this, contamination of the environment with insecticides has come to be considered hazardous because of carcinogenic and mutagenic effects [3,4], and other toxic effects on the skin, lung, mucous membrane [5], immune system, liver and blood [6,7], and the inhibition of protein synthesis in *Escherichia coli* [8]. Therefore, remediation of contaminated sites is currently underway in order to develop safe, convenient and economically feasible methods for pesticide detoxification.

Soil microflora have been suggested as a potential candidate for the detoxification of pesticides [9]. The soil, contaminated with pesticides, could be decontaminated using inoculation with specifically adapted microorganisms [10].

Some research on malathion bio-degradation has been carried out in Egyptian soils [11,12] and in Republic of Korea [8]. More often however, microbial attack and growth on wide ranges of

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organophosphorus insecticides as sole sources of carbon and energy have been previously reported [13–17].

In the present study, a number of local bacterial isolates capable of utilising and hydrolysing malathion in minimal media were isolated and identified. The efficiency of the isolate MSO-5 of *Bacillus thuringiensis* to metabolise malathion as a sole carbon and energy source was investigated.

Material and methods

Malathion

Malathion diethyl (dimethoxy thiophosphorylthio-succinate) was obtained from the Kafr El Zayat Company/Egypt with a water solubility of 130 mg/l, soluble in most organic solvents.

Isolation of malathion-degrading bacteria

The medium used for isolation of malathion-degrading bacteria was Luria-Bertani (LB), containing 10 g/l trypton, 5.0 g yeast extract, 5.0 g sodium chloride adjusted to pH 7. Water samples were collected from agricultural waste water contaminated with organophosphorus pesticides at Berket El-Sabaa region near Menofia Governorate/Egypt. Each sample was serially diluted and plated on Luria-Bertani (LB) agar, overlaid with 2.5×10^3 ppm malathion. Plates were incubated for 3 days at 30 °C and malathion tolerant bacteria were selected.

Identification and characterisation of isolated bacteria

Morphological and biochemical characterisation

Growing colonies were streaked on LB agar plates for characterisation and identification. The selected 5 different colonies MOS-1 to MOS-5 were restreaked on LB agar plates for further purification. The purified colonies were stained with Gram and endospore stain and then examined microscopically to determine the shape and spore-forming ability of the selected isolates. Biochemical and physiological identification were carried out as described [18]. Biochemical identification included the growth in 1, 5 and 7% NaCl; growth at pH 5 and 7 and temperature of 30 and 50 °C; growth in the presence of lysozyme; production of acid and gas from carbohydrates; and assimilation of different carbohydrates. Other tests such as catalase reaction, citrate utilisation, coagulase test, gelatin liquefaction, hydrogen sulphide production, methyl red test, indol production, ornithene decarboxylase production, nitrate reduction, oxidation activity, degradation of tyrosine, deamination of phenyl alanine, hydrolysis of starch and formation of indole were also used in the identification of the isolated bacteria. Identification was also confirmed using the Sensitive Auto Identification System at the National Cancer Institute, Cairo/Egypt.

Molecular characterisation

Molecular tools such as protein banding patterns of RAPD-PCR analysis were applied to characterise the selected isolates as described [19,20]. The total cellular proteins were electrophoretically separated on SDS-polyacrylamide gel and visualised by Coomassie blue stain as described [21]. Bacterial isolates under investigation were grown in 3 ml LB-broth. Cells were harvested and washed once with 1 ml of 0.5 M NaCl and 5 mM EDTA and boiled for 5 min at 95 °C just prior to electrophoresis.

DNA was extracted from bacterial cells using the method described by Sambrook et al. [22] with some modifications optimised for Gram-positive bacteria. RAPD-PCR was performed according to Williams et al. [23]. Amplification reaction was carried out using 50 μg genomic DNA, 0.5 μM primer (Operon Technologies, Alameda/USA), two units Taq DNA polymerase (Promega Corp., Madison, USA) and 0.2 mM dNTPs. PCR amplification was performed for 40 cycles after an initial denaturation step at 94 °C for 3 min. Samples were subjected to denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min and extension at 72 °C for 2 min. An additional extension step at 72 °C for 5 min was performed. The amplification products were resolved in a 1.5% agarose gel.

Degradation and residual determination of malathion by the local isolate MOS-5 of B. thuringiensis

Residual determination of malathion in MOS-5 inoculated media

The non-degraded residual malathion was monitored in liquid culture of MOS-5 through Gas Chromatography Spectrometry–Mass Spectra (GC/MS) analysis. In this assay conical flask containing M9 minimal salt medium and malathion (250 ppm) were inoculated with 5.6×10^8 cfu/ml of MOS-5 and incubated at 30 °C for 15 days. M9 minimal salt medium contains 0.64% $Na_2HPO_4 \cdot 7H_2O$, 0.15% KH_2PO_4, 0.025% NaCl and 0.05% NH_4Cl. To 800 ml sterile deionized water, 200 ml of M9 salts were added. The percentage of residual malathion was determined at 0, 3, 7, 10 and 15 days post inoculation. Samples of metabolites during growth of MOS-5 were transferred to test tubes and methylated using the method of Muan and Skaare [24]. Malathion was analysed and identified using (GC/MS).

Growth of bacterial isolates in liquid culture supplied with malathion

MOS-5 was inoculated into M9 minimal medium supplied with 250 ppm malathion as the sole carbon source. Malathion was dissolved in acetone (250 mg/300 μl) and added to 100 ml M9 media. Bacterial growth was estimated based on determination of viable cell counts per ml (CFU ml^{-1}).

Results

Isolation of malathion-degrading bacteria

Two types of bacterial colonies were isolated ("A" and "B") based on colony and cell shape, cellular protein profile on SDS-PAGE and genetic make up profile (RAPD-PCR) on agarose gel. Group "A", characterised by small and slimy colonies, contained isolates MOS-1, MOS-2, MOS-3 and MOS-4. Group "B", on the other hand, characterised by beige coloured matt appearance colonies contained isolate MOS-5. These 5 bacterial isolates were also able to grow in minimal salt medium supplied with 250 ppm malathion as sole source of carbon and energy.

Identification of isolated species

Five isolates were initially identified using their morphological, physiological and biochemical characteristics as described [18,25]. The 4 isolates MOS-1, MOS-2, MOS-3 and MOS-4 were identified as *Enterobacter aerogenes*, while MOS-5 was identified as *B. thuringiensis* via the production of crystal protein and its entomocidal activity against different cotton pests (data not shown).

Table 1 Physiological characteristics of local isolates MOS-1, MOS-2, MOS-3 and MOS-4.

Test	Reaction	Test	Reaction
Motility	+	Utilisation of	
Oxidase	–	Malonate	+
Ornithine decarboxylase	+	Citrate	+
Arginie dihydrolase	–	Adonitol	+
Lysine decarboxylase	+	Ketogluconate	–
Urea hydrolysis	–	Glycerol	+
Methyl red	–	Muo-inositol	+
Voges-Proskauer	+	Melebiose	+
Gelatin hydrolysis	–	Raffinose	+
O–F test[a]		L-Rhamnose	+
Oxidative	+	D-Sorbitol	+
Fermentative	+	Arabinose	+
		Maltose	+
		D-Mannitol	+
		Trehalose	+

[a] O–F: oxidation–fermentation.

Microscopic examination of the local isolates MOS-1, MOS-2, MOS-3 and MOS-4 revealed that these isolates are Gram negative, straight rods, non-spore-forming bacteria, motile by peritrichous flagella. Optimal temperature for growth is 30–37 °C. Isolates are facultative anaerobic, with both respiratory and fermentative metabolism. Further characteristics are given in Table 1. Isolate MOS-5 is a Gram-positive, rod-shaped and a spore-producing bacterium. Each cell contains only one centrally located oval endospore (Fig. 1). The sporulating cells produce crystalline inclusion bodies. Numerous biochemical and physiological tests were carried out. The isolate MOS-5 showed an optimal growth rate at 30 °C, pH 7.0 and no growth at 50 °C or below pH 5.0. The isolate produces acids from only glucose, tolerates 7% NaCl, hydrolyses starch and gelatine, reduces nitrate to nitrite, utilises citrate, degrades tyrosine, reacts positive for catalase, and resists lysocyme. This isolate is unable to produce acids from either mannitol or xylose and does not form indole or deaminate phenyl alanine.

Physiological and biochemical characteristics of the isolates MOS-1, MOS-2, MOS-3 and MOS-4 of E. aerogenes

The four isolates MOS-1, MOS-2, MOS-3 and MOS-4 showed the same physiological and biochemical characteristics. All yielded negative results with oxidase, arginie dihydrolase, urea hydrolysis, methyl red and gelatin hydrolysis tests, and positive results with ornithine decarboxylase, lysine decarboxylase, Voges-Proskauer and oxidation–fermentation tests. While they gave positive results for the utilisation of malonate, citrate, adonitol, glycerol, muo-inositol, melebiose, raffinose, L-rhamnose, sorbitol, srabinose, maltose, D-mannitol and trehalose, they yielded a negative response for the utilisation of ketogluconate.

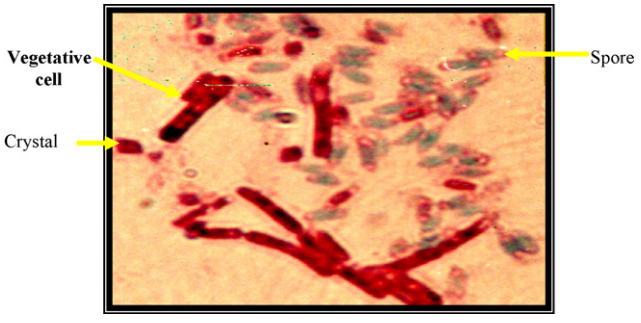

Figure 1 Photomicrograph of the local isolate MOS-5 showing oval central spores and crystal protein.

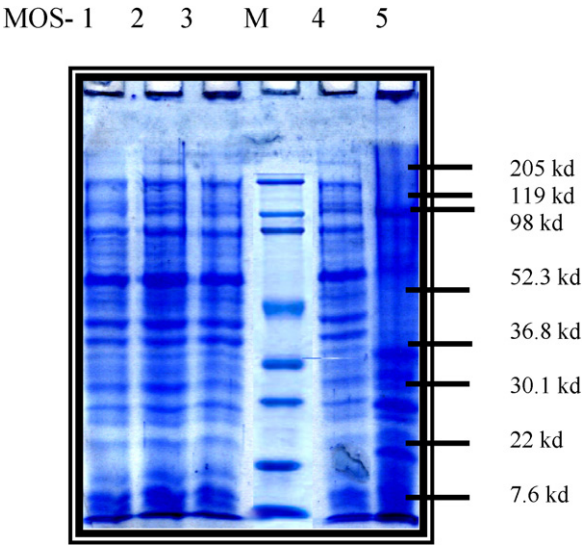

Figure 2 SDS-PAGE analysis of total cellular proteins of malathion-degrading local bacterial isolates stained with Coomassie brilliant blue lanes. (Numbers beside the gel indicate the molecular masses of standard marker protein. Protein banding patterns of total cellular proteins are shown above the lanes, which are marked with the abbreviation of each isolate MOS-1, MOS-2, MOS-3, MOS-4 and MOS-5.)

Molecular characterisation

Protein banding patterns

The total cellular proteins from vegetatively growing cells were fractionated on denaturing gel by electrophoresis (sodium dodecyl sulphate) SDS-polyacrylamide gel (Fig. 2). The protein binding patterns were identical in the four isolates MOS-1, MOS-2, MOS-3 and MOS-4 (Fig. 2, lanes 1–4). This finding indicates that these isolates are highly similar. MOS-5, on the other hand, showed a completely different pattern (Fig. 2, lane 5).

Total DNA profile

The difference between MOS-1, MOS-2, MOS-3 and MOS-4 could not be manifested at the protein banding level. Accordingly, the differentiation of these isolates was carried out at the DNA level. Random amplified polymorphic DNA (RAPD) analysis, using two operon primers (A_{17} and E_{18}), confirmed the results obtained by SDS-PAGE. Isolates MOS-1, MOS-2, MOS-3 and MOS-4 produced the same amplified DNA segments and were identical (Fig. 3A, lanes 1–8). In contrast, PCR-RAPD analysis of MOS-5 revealed its differences from the other isolates (Fig. 3B, lanes 9 and 10).

According to the obtained results, MOS-1, MOS-2, MOS-3 and MOS-4 were excluded from further experimental studies because isolates belonging to *E. aerogenes* are known to be the causative agent of urinary tract infection. Therefore, MOS-5 was selected for further study.

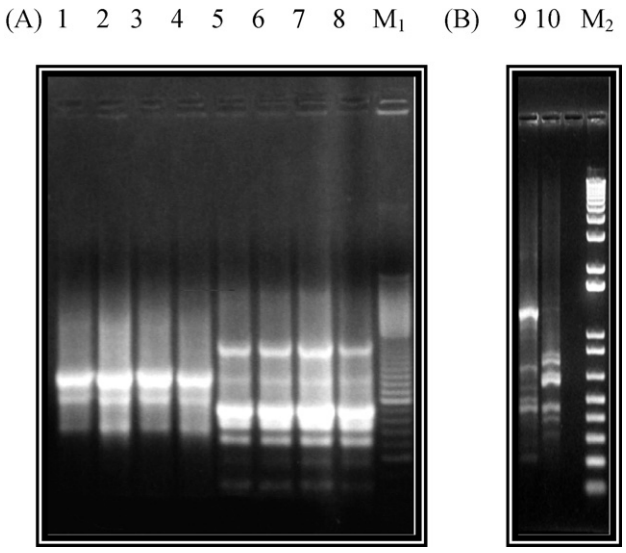

Figure 3 Ethidium bromide-stained agarose gel resolving RAPD-PCR profile of the five bacterial isolates (MOS-1, MOS-2, MOS-3, MOS-4 and MOS-5), amplified with RAPD primers Op-A_{17} and Op-E_{18}. M_1 and M_2 are DNA markers (M_1 is the 100 bp DNA ladder marker and M_2 is the 1 kb plus DNA ladder). Lanes 1–4 are MOS-1 to MOS-4 with Op-A_{17}. Lanes 5–8 are MOS-1 to MOS-4 with OP-E_{18}. Lanes 9 and 10 are MOS-5 with Op-A_{17} and Op-E_{18}, respectively.

Growth of B. thuringiensis (MOS-5) in liquid culture supplied with malathion

The results showed malathion supported growth of *B. thuringiensis* in M9 minimal medium supplied with 250 ppm malathion as a sole source of carbon after 12 days of incubation. The bacterial growth reached 7.87×10^{11} CFU ml^{-1}. A longer incubation period did not increase bacterial growth.

Degradation of malathion using the Egyptian isolate MOS-5 of B. thuringiensis

Malathion was the sole carbon source during growth of *B. thuringiensis* MOS-5 in a minimal salt medium containing 250 ppm malathion. The non-degraded residual malathion was monitored during 15 days incubation using GC/MS analysis. Slightly more than 50% of the initial malathion was decomposed within 3 days. The malathion concentration decreased to 17% in the inoculated medium after 10 days incubation, while more than 91% of the initial malathion was degraded after 15 days (Table 2).

Kamal et al. [26] identified the main metabolites in an aqueous fraction of culture filtrate of the isolate MOS-5 of *B. thuringiensis*. The results indicated that two major metabolites appeared during 7 days of incubation. HPLC and mass spectrometric analysis data revealed that the two principle metabolites produced from biodegradation of malathion are of mono- and di-acid derivatives.

Discussion

Organophosphorus insecticides like malathion are considered to be hazardous and have been known to potentially cause adverse effects on human health by inhibition of acetylcholinesterase activity in the body [27]. Therefore, remediation of contaminated sites is of general interest. It is very important to find a novel biocatalyst for degrading effectively organophosphorus insecticides in the environment.

Table 2 Percentage of recovery of residual malathion in free M9 minimal media in comparison with M9 media inoculated with MOS-5.

Incubation time (days)	% Recovery of residual malathion	
	MOS-5 free medium	MOS-5 inoculated medium
0	100	100
3	71.45	49.4
7	60.70	26.1
10	38.91	17.0
15	28.90	9
21	20.13	4
30	13.33	0.7

Five local malathion hydrolysing bacterial isolates, designated as MOS-1, MOS-2, MOS-3, MOS-4 and MOS-5, were obtained from agricultural waste water. These five isolates were capable of growing on minimal salt media containing 250 ppm malathion as a sole carbon source. The bacterial and fungal degradation and utilisation of similar compounds as sole carbon sources have been reported by others [8,9,11,13,16,28,29]. The five bacterial isolates under investigation were identified according to classical bacteriological methods [25] and, since the phenotypic characteristics of any organism are the translation of its genetic contents, advanced molecular techniques were used to examine the microbes at the genetic level.

Therefore, the examination of any microbe at the DNA level is more informative than the classical identification methods [20,30]. The examination of the protein pattern of these isolates indicated that these isolates belong to two different bacterial groups. Isolates MOS-1, MOS-2, MOS-3 and MOS-4 had an identical protein profile and differed from the isolate MOS-5. Due to the high difficulty of achieving differentiation between MOS-1, MOS-2, MOS-3 and MOS-4 using SDS-PAGE, RAPD-PCR was carried out according to Williams et al. [23] with minor modifications.

Data revealed that the four bacterial isolates MOS-1, MOS-2, MOS-3 and MOS-4 were identical. On the other hand, isolate MOS-5 was completely different and could be easily distinguished from the other isolate. Expectedly, data obtained from RAPD analysis confirmed those obtained from SDS-PAGE.

Bacterial isolates MOS-1, MOS-2, MOS-3 and MOS-4 were identified as *E. aerogenes*. Isolate MOS-5, on the other hand, was identified as *B. thuringiensis*. Interestingly, MOS-5 possesses high entomocidal activity against cotton pests such as cotton leaf worm (*Spodoptera littoralis*) and pink boll worm (*Pectinophera gossypiella*). Isolates MOS-1, MOS-2, MOS-3 and MOS-4 were excluded from further studies, because *E. aerogenes* is a pathogenic organism and is known as a causative agent of the urinary tract infection. Only MOS-5 was selected for further studies.

In the current study, the persistence rate of malathion in liquid culture of the isolate MOS-5 of *B. thuringiensis* grown in minimal salt medium containing malathion as the sole carbon and energy source was estimated during 15 days of incubation time. The obtained results revealed that a considerable removal of malathion after 3 days of incubation was observed. In inoculated salt media, for instance, more than 50% of the initial malathion was degraded to other compounds compared to non-inoculated media. After 1 week of incubation, residual malathion decreased to 26.5% and reached 9% after 15 days of incubation. On the other hand, residual

malathion in free salt media incubated for 15 days was reduced to 95% due to spontaneous degradation.

HPLC and mass spectrometric analysis revealed that the isolate MOS-5 of *B. thuringiensis* is very active in degrading malathion, probably through the action of carboxyl ester hydrolysis. Detoxification of several organophosphorus pesticides in the environment is carried out by carboxy esterase. Organophosphorus hydrolase enzymes catalyse the hydrolysis of a wide range of organophosphorus pesticides [31]. Different groups of these enzymes are found in bacteria [12,28]. Malathion degradation by cutinase and yeast esterase has been reported by Kim et al. [8].

A possible approach to the practical application of *B. thuringiensis* may be to develop a microbial gene expression system. With this the culture medium, when containing large amounts of extracellular recombinant hydrolytic enzymes, can be directly applied to the in situ degradation of malathion without costly purification.

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